# NOVEL METHOD OF DETECTING AMYLOID-LIKE FIBRILS OR PROTEIN AGGREGATES

## Related Applications

This application claims priority to PCT/EP98/04810, filed on July 31, 1998, which claims priority to European Patent Application 97113320.2, filed on August 1, 1997.

## Field of the Invention

The present invention relates to methods of detecting the presence of detergent or urea-insoluble amyloid-like fibrils or protein aggregates on filters. Preferably, said fibrils or aggregates are indicative of a disease, preferably of a neurodegenerative disease such as Alzheimer's disease or Huntington's disease. In addition, the present invention relates to inhibitors identified by the method of the invention, to pharmaceutical compositions comprising said inhibitors and to diagnostic compositions useful for the investigation of said amyloid-like fibrils or aggregates.

## Background of the Invention

Amyloid-like fibrils and aggregates are found widespread in nature. For example, protein aggregates are found as inclusion bodies in bacteria. Such inclusion bodies may in particular arise during the recombinant expression of proteins in bacteria. In addition, a variety of diseases, both in humans and animals, is characterized by the pathogenic formation of amyloid-like fibrils or protein aggregates in neuronal tissues. A well-known and typical example of such diseases is Alzheimer's disease (AD). AD is characterized by the formation of neurofibrillar tangles and ß-amyloid fibrils in the brain of AD patients. Similarly, scrapie is associated with the occurrence of scrapie-associated fibrils in brain tissue.

Another class of these diseases is characterized by an expansion of CAG repeats in certain genes. The affected proteins display a corresponding polyglutamine expansion. Said diseases are further characterized by a late onset in life and a dominant pathway of inheritance.

A typical representative of this class of diseases is Huntington's disease. Huntington's disease (HD) is an autosomal dominant progressive neurodegenerative disorder characterized by personality changes, motor impairment and subcortical dementia (Harper, 1991). It is associated with a selective neuronal cell death occurring primarily in the cortex and striatum (Vonsattel et al., 1985). The disorder is caused by a CAG/polyglutamine (polygln)

disease may be analyzed by complex methodology; see, for example, Booth et al. Nature 385 (1997), 787-793, and references cited therein.

So far, however, a simple method that may be established in any laboratory without sophisticated equipment is not available. Such a method would be especially useful in routine procedures such as the testing of patient samples for amyloid-like fibrils or protein aggregates. Accordingly, the technical problem underlying the present invention was to provide a rather simple method that may routinely be used for the detection of such fibrils or aggregates. The solution to said problem is provided by the embodiment characterized in the claims.

## Summary of the Invention

Thus, the present invention relates to a method of detecting the presence of detergent- or urea-insoluble amyloid-like fibrils or protein aggregates on a filter comprising the steps of (a) contacting said filter with material suspected to comprise said fibrils or aggregates and (b) detecting said fibrils or aggregates are retained on said filter.

In accordance with the present invention, it has surprisingly been found that filters of a variety of consistencies may be employed to retain detergent- or urea-insoluble amyloid-like fibrils or protein aggregates on their surface. Essentially, only the above-recited two steps are necessary in order to investigate whether said fibrils or aggregates are present in a sample.

The first step comprises contacting the filter with material suspected to comprise said fibrils or aggregates. The term "suspected to comprise" is intended to mean that the investigator may start from the assumption that the material indeed contains such fibrils or aggregates. Alternatively, said term means that it is totally unclear whether the material under investigation comprise such fibrils or aggregates.

It may be appropriate to pretreat the material prior to application to the filter. For example, for the detection of inclusion bodies, it may be desirable to first lyse the cells and set the cytoplasmic fraction free. Also, it may be useful to pretreat the patient samples prior to application to the filter. Said pretreatment may be effected, for example by employing proteases.

The detection of fibrils that are retained on the filter (the second step) may also be effected by a variety of steps. For example, detection may be effected by Western blot techniques, if an appropriate antibody is available.

Description of Figures, Sequences and Embodiments

**Figure 1**

**SDS-PAGE Analysis of Purified GST and GST-HD Fusion Proteins.**

(a) Aliquots (15 ml)of eluates from the gluatathione agarose column were subjected to 12.5% SDS-PAGE and analyzed by staining with Coomassie blue R. Lanes 1-6 contain GST, GST-HD20, -HD30, -HD83 and -HD122, respectively.

**Figure 2**

**Structure of GST-HD fusion proteins.**

The amino acid sequence corresponding to exon 1 of huntingtin is boxed. Arrows labeled Xa and T indicate cleavage sites for factor Xa and trypsin, respectively.

**Figure 3**

**Site-Specific Proteolysis of GST-HD Fusion Proteins with Trypsin and Factor Xa.**

Tryptic digestions were performed at 37°C for 3 (a) or 16 h (b). Native proteins and their cleavage products were subjected to 12.5% SDS-PAGE, blotted onto nitrocellulose membranes, and probed with anti-HD1 antibody. Arrows mark the origin of electrophoresis. (c) Purified fusion proteins and their factor Xa and trypsin cleavage products were analyzed using the filter retardation assay. The proteins retained by the cellulose acetate and nitrocellulose membranes were detected by incubation with the anti-HD1 antibody.

**Figure 4**

**Electron Micrographs of Native GST-HD Fusion Proteins and their Factor Xa and Trypsin Cleavage Products.**

Purified GST fusion proteins were protease treated, negatively stained with uranyl acetate and viewed by electron microscopy. The undigested GST-HD51 molecules appear as a homogeneous population of small, round particles (a).

Monoclonal anti-ß-amyloid, or the polyclonal anti-HD antibody, A1, A2, and A3: protein extracts prepared from cerebral cortex of Alzheimer's disease patients; C1, C2, and C3: protein extracts prepared from cerebral cortex of normal individuals. GST-HD51, fusion of glutathione S-transferase and huntingtin exon 1 containing 51 glutamines.

**Sequences**

SEQ ID NO: 1 is a primer (ES25) having the following sequence: TGGGATCCGC ATGGCGACCC TGGAAAAGCT GATGAAGG

SEQ ID NO: 2 is a primer (ES26) having the following sequence: GGAGTCGACT CACGGTCGGT GCAGCGGCTC CTCAGC

SEQ ID NO: 3 is a primer (ES27) having the following sequence: CTCCTCGAGC GGCGGTGGCG GCTGTTGCTG CTGCTGCTG

SEQ ID NO: 4 is a primer (BIO1) having the following sequence: CGCTCGAGGG TATCTTCGAG GCCCAGAAGA TCGAGTGGCG ATCACCATGA G

SEQ ID NO: 5 is a primer (BIO2) having the following sequence: GGCCGCTCAT GGTGATCGCC ACTCGATCTT CTGGGCCTCG AAGATACCCT CGAG

SEQ ID NO: 6 is a peptide having the following sequence: Ile Glu Gly Arg Gly Ile Arg Met Ala Thr Leu Glu Lys Leu Met Lys Ala Phe Glu Ser Leu Lys Ser Phe $(Gln)_n$ $(Pro)_{11}$ Gln Leu Pro Gln Pro Pro Pro Gln Ala Gln Pro Leu Leu Pro Gln Pro Gln $(Pro)_{10}$ Gly Pro Ala Val Ala Glu Glu Pro Leu His Arg Pro

SEQ ID NO: 7 is a peptide having the following sequence: Ile Glu Gly Arg Gly Ile Arg Met Ala Thr Leu Glu Lys Leu Met Lys Ala Phe Glu Ser Leu Lys Ser Phe $(Gln)_{20}$ Pro Pro Pro Pro Leu Glu Arg Pro His Arg Asp

SEQ ID NO: 8 is a peptide having the following sequence: Ile Glu Gly Arg Gly Ile Arg Met Ala Thr Leu Glu Lys Leu Met Lys Ala Phe Glu Ser Leu Lys Ser Phe $(Gln)_{51}$ Pro Pro Pro Pro Leu Glu Arg Pro His Arg Asp

SEQ ID NO: 9 is a peptide having the following sequence: Ile Glu Gly Arg Gly Ile Arg Met Ala Thr Leu Glu Lys Leu Met Lys Ala Phe Glu Ser Leu Lys Ser Phe $Gln_{20}$ Pro Pro Pro Pro Leu Glu Gly Ile Phe Glu Ala Gln Lys Ile Glu Trp Arg Ser Pro

SEQ ID NO: 10 is a peptide having the following sequence: Ile Glu Gly Arg Gly Ile Arg Met Ala Thr Leu Glu Lys Leu Met Lys Ala Phe Glu Ser Leu Lys Ser Phe $(Gln)_{51}$ Pro Pro Pro Pro Leu Glu Gly Ile Phe Glu Ala Gln Lys Ile Glu Trp Arg Ser Pro

The examples illustrate the invention:

**Example 1:**

**Purification of GST-HD fusion proteins containing expanded polyglns**

Exon 1 of the HD gene was isolated from genomic phage clones, derived from the normal and expanded alleles of an HD patient (Sathasivam et al., 1997), and used for the expression of GST-HD fusion proteins in *E. coli*. DNA fragments containing CAG repeats in the normal $(CAG)_{20-33}$ and expanded $(CAG)_{37-130}$ range were cloned into pGEX-5X-1 (Pharmacia), and the resulting plasmids expressing fusion proteins with 20 (GST-HD20), 30 (-HD30), 51 (-HD51), 83 (-HD83) and 122 (-HD122) glutamines, respectively, were used for protein purification. For plasmid construction lambda phage from stock $9197_4$ (Sathasivam et al., 1997) were plated single plaques which were innoculated into 400 ml cultures of *E. coli* XL1-Blue MRF' (Stratagene) for DNA preparation. The DNA sequence encoding the N-terminal portion of huntingtin (exon 1), including the CAG repeats, was amplified by PCR using the following pair of primers: ES 25 (TGGGATCCGCATGGCGACCCTGGAAAAGCTGATGAAGG) (Seq. ID No. 1) corresponding to nt315-343 of the HD gene (HDCRG, 1993) and containing a BamHI site (underlined) and ES 26 (GGAGTCGACTCACGGTCGGTGCAGC GCTCCTCAGC) (Seq. ID No. 2) corresponding to nt516-588 and containing a Sall site (underlined). Conditions for PCR were as described (Mangiarini et al. 1996). Due to instability of the CAG repeat during propagation in *E. coli*, DNA preparations from individual plaques yielded different sized PCR products. Fragments of ~320, 360, 480, and 590 bp were gel-purified, digested with BamHI and Sall and inserted into the BamHI-Sall site of the expression vector pGEX-5X1 (Pharmacia) yielding pCAG30, pCAG51, pCAG83 and pCAG122, respectively. PCAG20, containing 20 repeats of CAG within the cloned HD exon 1 sequence, was similarly constructed from a phage genomic clone derived from a normal allele. All constructs were verified by sequencing. After induction with IPTG, the resulting proteins were purified under native conditions by affinity chromatography on glutathione agarose. Thus, *E. coli* SCS1 (Stratagene) carrying the pGEX expression plasmid of interest was grown to an $OD_{600nm}$ of 0.6 and induced with IPTG (1 mM0 for 3.5 h as described in the manufacturer's protocol (Pharmacia). Cultures (200 ml) of induced bacteria were centrifuged at 4000 g for 20 min, and the resulting pellets were stored at -

a Nll from a 17 month old R6/5 homozygous mouse is shown in Fig. 6c. This Nll (large arrow) contains high molecular weight fibrous structures which were clearly differentiated from the surrounding chromatin. The filaments were randomly oriented, 5-10nm in diameter and often measured up to 250 nm in length (small arrows). These structures differ from those previously reported in the Nlls seen in hemizygous R6/2 mice which were far more granular in composition, with individual filamentous structures being more difficult to distinguish (Davies et al., 1997). R6/2 mice exhibit an earlier age of onset with a more rapid progression of the phenotype and do not survive beyond 13 weeks (Mangiarini et al., 1996). It is possible that the filamentous structures do not have time to form in the R6/2 mice.

**Example 5:**

**Construction of further plasmids, purification of corresponding GST fusion Proteins and proleolytic cleavage of GST fusion proteins**

In a second set of experiments, a further number of plasmids was constructed. Standard protocols for DNA manipulations were followed (J. Sambrook, E.F. Fritsch, and T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Plainview, NY, 1989). *IT*-15 cDNA sequences (HDCRG, *Cell* **72**, 971 (1993)) encoding the N-terminal portion of huntingtin, including the CAG repeats, were amplified by PCR using the oligonucleotides ES25 (5'-TGGGATCCGCATGGCGACCCTGGAAAAGCTGATGA AGG-3') (Seq. ID No. 1) and ES27 (3'-CTCCTCGAGCGGCGGTGGCGGCTGTTGCTG CTGCTGCTG-5') (Seq. ID No. 3) as primers and the plasmids pCAG20 and pCAG51 as template (E. Scherzinger, R. Lurz, M. Trumaine, L. Margiarini, B. Hollenbach, R. Hasenbank, G. P. Bates, S. W. Davies, H. Lehrach, and E. E. Wanker, *Cell* **90**, 549 (1997)). Conditions for PCR were as described (L. Mangiarini, K. Sathasivam, M. Seller, B. Cozens, A Harper, C. Hetherington, M. Lawton, Y Trottier, H. Lehrach, S. W. Davies, and G. P. Gates, *Cell* **87**, 493 (1996)). The resulting cDNA fragments were gel purified, digested with *Bam* HI and Xho I and were inserted into the *Bam* HI-*X*ho I site of the expression vector pGEX-5X-1 (Pharmacia), yielding pCAG20DP and pCAG51DP, respectively. The plasmids pCAG20DP-Bio and pCAG51DP-Bio were generated by subcloning the PCR fragments obtained from the

plasmids pCAG20 and pCAG51 into pGEX-5X-1-Bio. PGEX-5X-1-Bio was created by ligation of the oligonucleotides BIO1 (5'-CGCTCGAGGGTATCTTCGAGGCCCAGAAGATCGAGTG GCGATCACCATGAG-3') (Seq. ID No. 4) and BIO2 (5'-GGCCGCTCATGGTGATCGCC ACTCGATCTTCTGGGCCTCGAAGATACCCTCGAG-3') (Seq. ID No. 5), after annealing and digestion with *Xho* I, into the *Xho* I-*Not* I site of pGEX-5X-1. The plasmids with the *IT*-15 cDNA inserts were sequenced to confirm that no errors had been introduced by PCR. The construction of plasmids pTL1-CAG20, pTL1-CAG51 and pTL1-CAG93 for the expression of huntingtin exon 1 proteins containing 20, 51 and 93 glutamines in mammalian cells has been described (A. Sittler, S. Walter, N. Wedemeyer, R. Hasenbank, E. Scherzinger, G. P. Bates, H. Lehrach, and E. E. Wanker, *Mol. Cell*, submitted).

Cont A5

The amino acid sequence of the GST-HD fusion proteins encoded by the *E. coli* expression plasmids pCAG20DP, pCAG51DP, pCAG20Dp-Bio and pCAG51DP-Bio is shown in Fig. 8. The plasmids pCAG20DP and pCAG51DP encode fusion proteins of glutathione S-transferase (GST) and the N-terminal portion of huntingtin containing 20 (GST-HD20DP) and 51 (-HD51DP) polyglutamines, respectively. In these proteins the proline-rich region located immediately downstream of the glutamine repeat was deleted (E. Scherzinger, R. Lurz, M. Trumaine, L. Margiarini, B. Hollenbach, R. Hasenbank, G. P. Bates, S. W. Davies, H. Lehrach, and E. E. Wanker, *Cell* **90**, 549 (1997). The fusion proteins GST-HD20DPBio and -HD51DPBio are identical to GST-HD20DP and -HD51DP, except for the presence of a biotinylation site (P. J. Schatz, *Biotechnology* **11**, 1138 (1993)) at their C-termini.

In the experiments described herein, *E. coli* DH10B (BRL) was used for plasmid construction and *E. coli* SCS1 (Stratagene) was used for the expression of GST-HD fusion proteins. Transformation of *E. coli* with plasmids and ligation mixtures was performed by electroporation using a Bio-Rad Gene Pulser (Richmond, CA). Transformed cells were spread on LB plates supplemented with appropriate antibiotics (J. Sambrook, E. F. Fritsch, and T. Maniatis, Molecular Clone: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Plainview, NY 1989). For expression of GST fusion proteins, cells were grown in liquid TY medium

Smith, D. B., and Johnson, K. S. (1988). Single-step purification of peptides expressed in *Eschedrichia coli* as fusions with glutathione S-transferase. Gene *67*, 31-40.

Stott, K., Blackgurn, J. M., Butler, P. J. G., and Perutz, M. (1995). Incorporation of glutamine repeats makes protein oligomerize: implications for neurodegnerative diseases. Proc. Natl. Acad. Sci. USA *92*, 6509-6513.

Tellez-Nagel, I., Johnson, B., and Terry, R. D. (1974). Studies on brain biopsies of patients with Huntington's chorea. J. Neurocyt. *3*, 308-332.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA *76*, 4350-4354.

Trottier, Y., Devys, D., Imbert, G., Sandou, F., An, I., Lutz, Y., Weber, C., Agid, Y., Hirsch, E. C., and Mandel, J.-L. (1995a). Cellular localisation of the Huntington's disease protein and discrimination of the normal and mutated forms. Nature Genet. *10*, 104-110.

Trottier, Y., Lutz, Y., Stevanin, G., Imbert, G., Devys, D., Cancel, G., Sandou, F., Weber, C. David, G., Tora, L., Agid, Y., Brice, Al., and Mandel, J.-L. (1995b). Polyglutamine expansion as a pathological epitope in Huntington's disease and four dominant cerebellar ataxias. Nature, *378*, 403-406.

Vonsattel, J.-P, Myers, R.H., Stevens, T. J., Ferrante, R. J., Bird, E. D., and Richardson, E. P. (1985). Neuropathological classification of Huntington's disease. J. Neuropath. Exap. Neurol. *44*, 559-577.

Wanker, E. E., Rovira, C., Scherzinger, E., Hasenbank, R., Walter, S., Tait, D., Colicelli, J., and Lehrach, H. (197). HIP-1: A huntingtin interacting protein isolated by the yeast two-hybrid system. Hum. Mol. Genet. *6*, 487-495.

We claim: